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Competition and Coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the Dental Biofilm

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The human mucosal surface is colonized by the indigenous microflora, which normally maintains an ecological balance among different species. Certain environmental or biological factors, however, may trigger disruption of this balance, leading to microbial diseases. In this study, we used two oral bacterial species, *Streptococcus mutans* and *Streptococcus sanguinis* (formerly *S. sanguis*), as a model to probe the possible mechanisms of competition/coexistence between different species which occupy the same ecological niche. We show that the two species engage in a multitude of antagonistic interactions temporally and spatially; occupation of a niche by one species precludes colonization by the other, while simultaneous colonization by both species results in coexistence. Environmental conditions, such as cell density, nutritional availability, and pH, play important roles in determining the outcome of these interactions. Genetic and biochemical analyses reveal that these interspecies interactions are possibly mediated through a well-regulated production of chemicals, such as bacteriocins (produced by *S. mutans*) and hydrogen peroxide (produced by *S. sanguinis*). Consistent with the phenotypic characteristics, production of bacteriocins and H_2O_2 are regulated by environmental conditions, as well as by juxtaposition of the two species. These sophisticated interspecies interactions could play an essential part in balancing competition/coexistence within multispecies microbial communities.

The human mucosal surface is colonized by large numbers of bacterial species, the so-called indigenous flora (14, 18, 36). In a homeostatic state, this indigenous flora plays an important role in protecting the host from invasions by exogenous pathogens; however, when the homeostasis is disrupted, it can cause diseases, such as dental caries, periodontal disease (35), vaginitis (10), and inflammatory bowel disease (23). Understanding the molecular mechanisms through which interspecies interactions can lead to homeostasis would shed new light on the development of novel measures to curb these "polymicrobial" diseases. In this study, we used two members of the dental biofilm, *Streptococcus mutans* and *Streptococcus sanguinis* (formerly *S. sanguis*) (22), as a model to investigate the interspecies interactions leading to competition and coexistence.

The dental biofilm is a good model system for studying interspecies interactions owing to its vast biodiversity (>500 bacterial species) (17, 26, 30), high cell density (~10^{11} cells/g [wet weight]) (9), and easy accessibility (29). In addition, the oral cavity is an environment with constant cycles of feast and famine and fluctuations of pH due to food intake from the host. The high density and diversity of oral biofilm community members coupled with a limited food supply should create an environment that is conducive to fierce competition for available resources.

S. mutans is considered a major pathogen causing human dental caries (also known as tooth decay) (19). *S. mutans* normally exists as a regular member of the mature dental biofilm community; however, under certain conditions, it can become dominant to cause dental caries (21). *S. sanguinis* is also a member of the oral biofilm community (28). Except for reported associations with bacterial endocarditis (37), *S. sanguinis* is considered a benign, or even a beneficial, bacterium with regard to dental caries (2, 5). The antagonism between *S. mutans* and *S. sanguinis* at the ecological level has been known for many years. Epidemiological studies showed that early colonization and high levels of *S. sanguinis* in an infant's oral cavity correlate with significantly delayed colonization by *S. mutans* (5). Similarly, high levels of *S. mutans* in the oral cavity correlate with low levels of *S. sanguinis* (20). Early studies with germ-free rats also demonstrated a so-called "competitive exclusion" between *S. mutans* and *S. sanguinis* depending on the sequence of inoculation (25). Despite these interesting early findings, no further studies were conducted to understand the molecular mechanisms underlying these interspecies interactions. In this study, we developed several new cellular assays for more defined analyses of the competition and coexistence between *S. mutans* and *S. sanguinis*. Our results, obtained by using a combination of physiological, genetic, and biochemical approaches, led us to propose a possible molecular mechanism underlying these fascinating interspecies interactions.

MATERIALS AND METHODS

Bacterial strains, media, and enzymes. *S. mutans* UA140 (32) and derivative strains constructed in this study are listed in Table 1. *S. sanguinis* ATCC 10556 was used for competition analysis. Other streptococcal species used in the initial screening were *S. gordonii*, *S. pyogenes*, *S. oralis* ATCC 10557, *S. mitis* ATCC 33399, *S. mitis* ATCC 903, *S. pneumoniae*, *S. cristatus* ATCC 49999, *S. parasanguinis* ATCC 15911, *S. sanguinis* NY101, and *S. sobrinus* OMZ176. All species were routinely grown in brain heart infusion (BHI) medium (Difco, Sparks, MD) or on BHI plates under anaerobic conditions (90% N_2, 5% CO_2, 5% H_2) at 37°C unless otherwise indicated. Peptidase, hydrogen peroxide (30% [wt/wt]), and horseradish peroxidase were from Sigma (St. Louis, MO).

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Reference
S. sanguinis ATCC 10556	Oral commensal	
S. mutans UA 140	Wild-type MutI$^+$IV$^+$	32
UA140I$^-$IV$^+$	Δ*mutC* MutI$^-$IV$^+$	This study
UA140I$^+$IV$^-$	Δ*nlmAB* MutI$^+$IV$^-$	This study
UA140I$^-$IV$^-$	Δ*mutC* Δ*nlmAB* MutI$^-$IV$^-$	This study
UA140::Φ(*mutAp-luc*)	Φ(*mutAp-luc*) MutI$^+$IV$^+$	This study
UA140::Φ(*mutAp-mrfp*)	Φ(*mutAp-mrfp*) MutI$^+$IV$^+$	15
UA140::Φ(*ldhp-luc*)	Φ(*ldhp-luc*) MutI$^+$IV$^+$	24
UA140::Φ(*ldhp-gfp*)	Φ(*ldhp-gfp*) MutI$^+$IV$^+$	This study
UA140::Φ(*nlmAp-luc*)	Φ(*nlmAp-luc*) MutI$^+$IV$^+$	This study

Competition assays on plates and in biofilms. For competition assays on plates between *S. mutans* and *S. sanguinis*, 10 μl of an overnight culture of either species adjusted to an optical density at 600 nm (OD_{600}) of ~0.5 in 50% BHI was inoculated on half-strength BHI plates as the early colonizer. After an overnight incubation, 10 μl of the competing species at the same OD_{600} was inoculated beside the early colonizer as the later colonizer, or both species were inoculated at the same time beside each other. The plates were further incubated at 37°C anaerobically overnight before cell growth was inspected. For competition assays in biofilms, overnight cultures of *S. mutans* or *S. sanguinis* were diluted 1:100 in 50% BHI plus 0.1% sucrose and inoculated into a slide chamber. The cultures were incubated at room temperature for 3 h to allow cell attachment before the competing species was inoculated, or both species were inoculated at the same time. The biofilm was grown for 16 h at 37°C as a static culture. CellTracker Orange (Molecular Probes, Eugene, OR) was used to label all cells for 2 h before confocal microscopy. Confocal microscopy was performed as described previously (15).

Luciferase and mutacin production assays. Luciferase assays were performed as previously described (16). For planktonic culture, 100 μl of cell culture was used; for plate culture, cells were scraped from the plate and resuspended in 100 μl of BHI. The production of mutacin on the plate was measured by the antagonistic assay as described previously (32). Briefly, the plates were overlaid with the indicator strain *S. sobrinus* OMZ176 in a 1:5 dilution of an overnight culture in soft agar. After further incubation, the cleared zone was measured.

Assays for H_2O_2 production in liquid and on plate cultures. The production of H_2O_2 by *S. sanguinis* in liquid culture was measured as described previously (27). To measure the effect of *S. mutans* on the H_2O_2 production of *S. sanguinis*, an overnight culture of *S. sanguinis* was diluted to ~10^7 cells/ml (OD_{600}, ~0.025) and incubated anaerobically at 37°C. After two doubling times, the cells were washed twice with BHI and the OD_{600} was adjusted to 0.2. One milliliter of the cell suspension was transferred to a tube, and 1 ml of either BHI or *S. mutans* UA140::Φ(*mutAp-luc*) cell suspension (OD_{600} = ~0.2) was added. The cells were further incubated either as a planktonic culture or as a cell pellet with medium (16,000 × *g* for 1.5 min) for 2 h before the H_2O_2 concentration was measured with the culture supernatant. For the determination of H_2O_2 production on the plate, 10 μl of peroxidase (64 μg) was added to a half-strength BHI plate containing 1 mg/ml leuco crystal violet. After the liquid was absorbed into the agar, 5 μl of *S. sanguinis* was inoculated at the same spot. After overnight incubation with a subsequent 2-h air exposure, the plate was inspected for the development of a purple color on and around the colony.

Construction of mutacin-defective strains. *S. mutans* strain UA140 produces two major mutacins, the lantibiotic mutacin I and the nonlantibiotic mutacin IV (32). To study the role of each mutacin in interspecies competition, we constructed three derivative strains defective in either mutacin I (UA140I$^-$IV$^+$), mutacin IV (UA140I$^+$IV$^-$), or both (UA140I$^-$IV$^-$). To construct UA140I$^-$IV$^+$, the tetracycline (Tet) resistance gene *tetM* from Tn*916* (7) was amplified by PCR and cloned into pCR2.1 cloning vector (Invitrogen). A DNA fragment encompassing 1 kb upstream and downstream of *mutC* (32) was amplified by PCR and cloned into pCR2.1 to form pCRBCD. To delete *mutC*, an inverse PCR was performed by using two primers, BR1 and DF1, both of which had a StuI restriction site incorporated at their 5′ ends. The *tetM* gene cassette was released from pCR2.1 by cutting with StuI restriction enzyme, whose recognition sequence was also incorporated into the primers for amplifying *tetM*, and inserted into pCRBCD at the same restriction site. The resulting plasmid was digested with PstI and SphI and transformed into UA140. The deletion construct was integrated into the chromosome by double-crossover homologous recombination. The transformants were selected on Tet plates (10 μg/ml). Ten independent transformants were randomly selected, tested, and confirmed for no production of mutacin I by the deferred-antagonism assay (8) using *S. sobrinus* OMZ176, which is sensitive only to mutacin I. The deletion construction in the mutacin I gene locus was further confirmed by PCR and genetic complementation. To construct UA140I$^+$IV$^-$, the same strategy was used, except that the kanamycin resistance gene *aphIII* (38) was used. The deletion mutation was also confirmed by PCR and genetic complementation. Since no indicator strain was sensitive only to mutacin IV, the defect in mutacin IV production was further confirmed by mutacin isolation under conditions in which mutacin I was not produced (32). Briefly, mutacin IV was isolated from the culture supernatant by extraction with an equal volume of chloroform from a wild-type strain and the mutacin IV-defective strain. For purification, the crude extract was applied to a Source 15RPC column and eluted with a gradient of buffers A (0.1% trifluoroacetic acid) and B (0.085% trifluoroacetic acid in 60% acetonitrile) using an LKB Purifier (Amersham Pharmacia Biotech, Piscataway, N.J.). The activity of the purified peptide was tested in an overlay assay with the indicator strain *S. sanguinis* NY101. Ten microliters each of different serial dilutions were spotted onto a BHI agar plate and, after they dried, overlaid with BHI soft agar (0.7% agar) containing the indicator strain *S. sanguinis* NY101. The activity was monitored by the occurrence of a cleared zone. To construct the double-mutant strain UA140I$^-$IV$^-$, chromosomal DNA was isolated from strain UA140I$^+$IV$^-$ and transformed into strain UA140I$^-$IV$^+$. Ten independent transformants were selected on Tet-plus-kanamycin plates and tested, and the lack of mutacin production was confirmed by using the indicator strain NY101, which was sensitive to both mutacins (32). The mutants were further confirmed by PCR.

Construction of reporter strains. The mutacin I promoter-luciferase reporter strain UA140::Φ(*mutAp-luc*) was constructed essentially as described previously (15), except that pFW5-luc (31) was used as the backbone plasmid. The lactate dehydrogenase (*ldh*) promoter-green fluorescent protein (*gfp*) reporter strain UA159::Φ(*ldhp-gfp*) was constructed in the same manner as described previously (16), except that the luciferase gene was replaced with *gfp*. Reporter strains were confirmed by PCR, as well as by spectinomycin resistance (800 μg/ml).

Inhibition assays with H_2O_2 and mutacin. To assay the inhibition of *S. mutans* by H_2O_2, a fresh overnight culture of strain UA140 was diluted 25-fold in fresh BHI. After 2.5 doubling times, the culture was divided and treated with different concentrations of H_2O_2 (0.0005%, 0.0025%, and 0.005%). The growth inhibitions were monitored by following the OD_{600} at indicated time points (see Fig. 4A). The inhibition of *S. sanguinis* was tested with purified mutacin I and mutacin IV. Mutacins I and IV were purified as described above and earlier (33). The activities of the individual peptides were tested in an overlay assay as described above with the indicator strain *S. sanguinis* NY101.

Expression of *mutAp-luc* and *ldhp-luc* under biofilm conditions. Overnight cultures of strains UA140::Φ(*ldhp-luc*), UA140::Φ(*mutAp-luc*) and *S. sanguinis* were adjusted to an OD_{600} of 1. Ten microliters of strain UA140::Φ (*ldhp-luc*) or UA140::Φ(*mutAp-luc*) alone or mixed in a 1:1 ratio with *S. sanguinis* was spotted onto a BHI plate. After 6 h of incubation, the cells were scraped from the plate and the luciferase activity was determined. The activity was normalized by the cell counts of *S. mutans* after serial dilution.

RESULTS

Characterization of interspecies competition between *S. mutans* and other oral streptococcal species. To get a global view of how prevalent interspecies competition is between *S. mutans* and other oral streptococci, we analyzed the inhibitory spectrum of *S. mutans* strain UA140 against 11 streptococcal species, including members of the mitis, mutans, viridans, and pyogenic groups: *S. gordonii* ATCC 10558, *S. oralis* ATCC 10557, *S. mitis* ATCC 33399, *S. mitis* ATCC 903, *S. pneumoniae*, *S. parasanguinis* ATCC 15911, *S. sanguinis* ATCC 10556, *S. sanguinis* NY101, *S. sobrinus* OMZ176. *S. cristatus* ATCC 49999, and *S. pyogenes.* UA140 was inoculated onto BHI plates and grown for 24 h before the other species were inoculated nearby. As shown in Fig. 1, *S. mutans* could inhibit the growth of all tested strains; however, the growth inhibition was less severe against *S. sobrinus*, a member of the mutans group. Based on this result, *S. sanguinis* was chosen for further

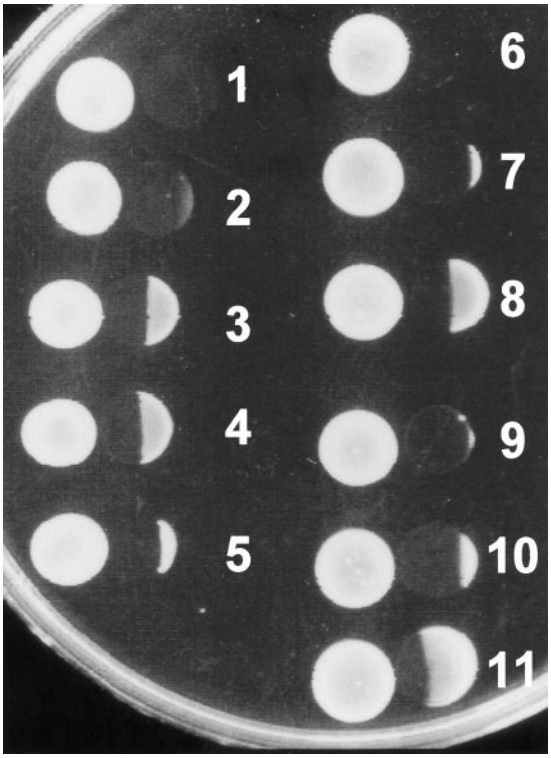

FIG. 1. Inhibition of oral streptococcal species by *S. mutans* UA140. 1, *S. gordonii*; 2, *S. pyogenes*; 3, *S. oralis*; 4, *S. mitis* ATCC 33399; 5, *S. mitis* ATCC 903; 6, *S. pneumoniae*; 7, *S. cristatus*; 8, *S. parasanguinis*; 9, *S. sanguinis* ATCC 10556; 10, *S. sanguinis* NY101; 11, *S. sobrinus*.

analysis because of its well-known history of antagonism toward *S. mutans*.

Competition between *S. mutans* and *S. sanguinis* in time and space. A simple competition assay was developed to test the antagonistic interactions between *S. mutans* and *S. sanguinis*. Overnight cultures of *S. mutans* UA140 and *S. sanguinis* ATCC 10556 were inoculated on half-strength BHI plates. Three tests were conducted: (i) *S. mutans* was inoculated first and allowed to grow overnight (as the early colonizer) before *S. sanguinis* was inoculated nearby (as the later colonizer), (ii) vice versa, and (iii) both species were inoculated at the same time. As shown in Fig. 2A, the early colonizer always inhibited the growth of the later colonizer regardless of the bacterial species (left and middle). This competitive exclusion was reduced to a negligible level when both species were inoculated at the same time (right). This suggested that the sequence of inoculation determined the competition outcome.

Since competitive exclusion could result from either nutritional deprivation by the growth of the early colonizer or production of inhibitory substances by the early colonizer, we decided to test the first possibility by performing the same competition assay described above but using different strains of the same species. We reasoned that nutritional deprivation would be more severe within the same species because the bacteria have the same nutrient requirements. We used another *S. mutans* strain, UA159 (1), in the competition assay with UA140 and another *S. sanguinis* strain, NY101, in the competition assay with ATCC 10556. No growth inhibition was observed in either competing pair regardless of the sequence of inoculation (data not shown). This result suggested that some diffusible substance produced by *S. mutans* and *S. sanguinis* rather than nutrient deprivation was responsible for the observed competitive exclusion.

To see if this competitive exclusion also occurred in space, such as in biofilms, we constructed an *S. mutans* green fluorescent protein (*gfp*) reporter strain, UA140::Φ(*ldhp-gfp*) (see Materials and Methods). UA140::Φ(*ldhp-gfp*) carries a *gfp* fusion to the lactate dehydrogenase (*ldh*) promoter on the chromosome. Since the *ldh* promoter is constitutively expressed (24), UA140::Φ(*ldhp-gfp*) cells continually exhibit green fluorescence throughout growth. This property made it easier to distinguish *S. mutans* from *S. sanguinis*, which was labeled with red fluorescence using a cell tracker dye (CellTracker Orange) 2 h prior to microscopy. UA140::Φ(*ldhp-gfp*) and *S. sanguinis* were then subjected to the previously described competition assays (see Materials and Methods). As shown in Fig. 2B, when *S. mutans* attached first, almost no *S. sanguinis* bacteria could attach and grow in the biofilm (left). The same was true for *S. sanguinis* when it attached first (middle). However, when both were inoculated at the same time, a mixed-species biofilm could form (right). This result was reminiscent of the observations made by Mikx et al. nearly 30 years ago in the germ-free-rat experiment (25), suggesting that the competition between *S. mutans* and *S. sanguinis* observed in this in vitro assay may also occur in vivo.

Environmental conditions modulate competition and coexistence between *S. mutans* and *S. sanguinis*. Since the dental biofilm in nature is continually challenged by adverse conditions, such as cycles of feast and famine and fluctuations of pH, we were interested to see whether the competition between *S. mutans* and *S. sanguinis* was influenced by these environmental conditions. We performed a plate assay similar to that shown in Fig. 2A under three conditions: a "nutrient-rich" growth condition in which sucrose was added to BHI and the medium was buffered to pH 7.0 with phosphate buffer, a "stress" condition in which the pH of BHI was lowered to 5.5, and a "nutrient-limiting" condition in which BHI was diluted to half strength, as in Fig. 2A. As expected, the "nutrient-limiting" condition resulted in the same pattern of inhibition shown in Fig. 2A. Surprisingly, under "nutrient-rich" or "stress" conditions, there was negligible or no inhibition between the species regardless of the sequence of inoculation (Fig. 2C and D); the lesser growth of *S. sanguinis* under "stress" conditions is due to the growth inhibition of *S. sanguinis* by acidic pH. These results suggested that environmental conditions modulated competition/coexistence between bacterial species.

Investigation of possible inhibitory substances produced by *S. mutans* and *S. sanguinis*. The results presented in Fig. 2A suggested that both *S. mutans* and *S. sanguinis* produced diffusible substances that inhibited the growth of the other species. To identify the possible inhibitory substances, we grew *S. mutans* and *S. sanguinis* on a half-strength BHI plate for 24 h and applied peroxidase (40 μg), peptidase (64 μg), or phosphate-buffered saline beside each colony for 10 min before the other species was inoculated at the same spot. The two enzymes (peptidase and peroxidase) were chosen based on previous knowledge that proteinaceous inhibitory substances (8)

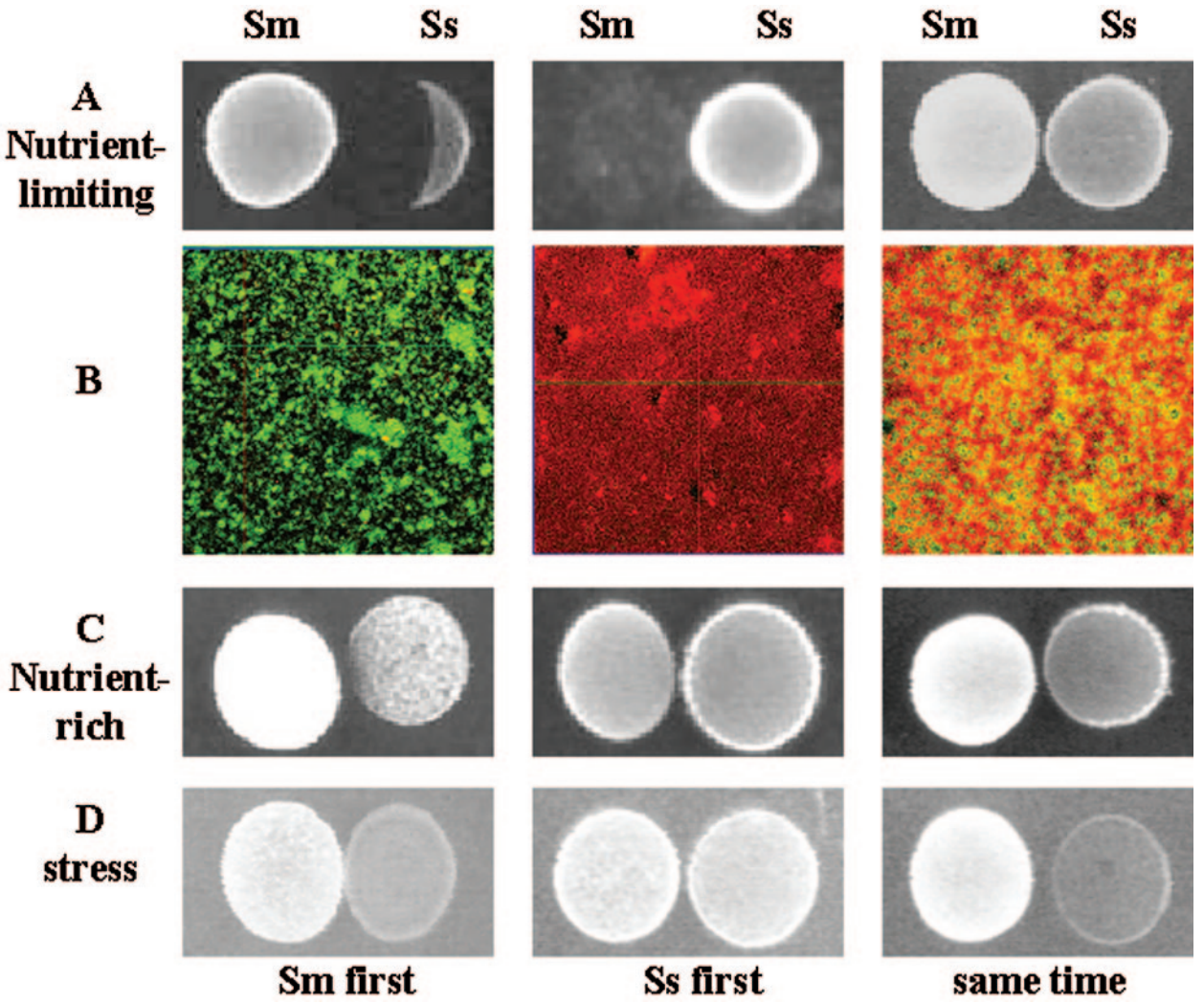

FIG. 2. Competition assays between *S. mutans* and *S. sanguinis*. (A) Competition assay on half-strength BHI plate. (B) Confocal laser scanning microscopy analysis of competition in biofilms. Green cells, *S. mutans* (green fluorescent protein); red cells, *S. sanguinis* (Cell-tracker orange). The pictures were taken at ×100 magnification. (C) competition assays on "nutrient-rich" plate (BHI plus 1% sucrose, buffered to pH 7). (D) competition assays on "stress" plate (BHI at pH 5.5). (C and D) Left, *S. mutans* (Sm) was inoculated first; middle, *S. sanguinis* (Ss) was inoculated first; right Sm and Ss were inoculated at the same time.

and H_2O_2 were produced by oral streptococci (34, 39). As shown in Fig. 3, addition of peroxidase abolished the inhibitory effect of *S. sanguinis* toward *S. mutans* (Fig. 3A, left), while addition of peptidase diminished the inhibitory effect of *S. mutans* toward *S. sanguinis* (Fig. 3B, middle). Given the fact that the inhibitory substance(s) produced by *S. mutans* is proteinaceous, one logical candidate would be a peptide antibiotic, e.g., bacteriocin, since *S. mutans* is known to produce multiple bacteriocins called mutacins (8). Strain UA140, used in this study, was known to produce two major mutacins, mutacin I and mutacin IV (32). To determine whether the mutacins were responsible for inhibiting the growth of *S. sanguinis*, we constructed a mutacin-defective isogenic strain, UA140I$^-$IV$^-$, in which the production of both mutacins was eliminated by inactivation of the mutacin-biosynthetic genes (see Materials and Methods). This mutant strain was tested in competition assays with *S. sanguinis* on the plate, as well as in the biofilm. As shown in Fig. 3C and D, UA140I$^-$IV$^-$ could no longer inhibit the growth of *S. sanguinis* on the plate or in the biofilm even when it was inoculated first. Similar results were obtained with all 11 oral streptococci used in the initial screen (data not shown). To test which mutacin was responsible for the inhibitory effect, UA140 derivative strains defective in either mutacin I or mutacin IV were constructed (see Materials and Methods). Competition assays using these strains showed that they were still able to inhibit the growth of *S. sanguinis* (data not shown). These results demonstrate that both mutacins serve as inhibitory substances and that either mutacin is sufficient to inhibit the growth of *S. sanguinis* and other streptococcal strains.

Since the inhibitory substance(s) produced by *S. sanguinis* was sensitive to peroxidase (Fig. 3A), hydrogen peroxide (H_2O_2), became the likely candidate. To test this hypothesis, we used a leuco crystal violet assay (see Materials and Meth-

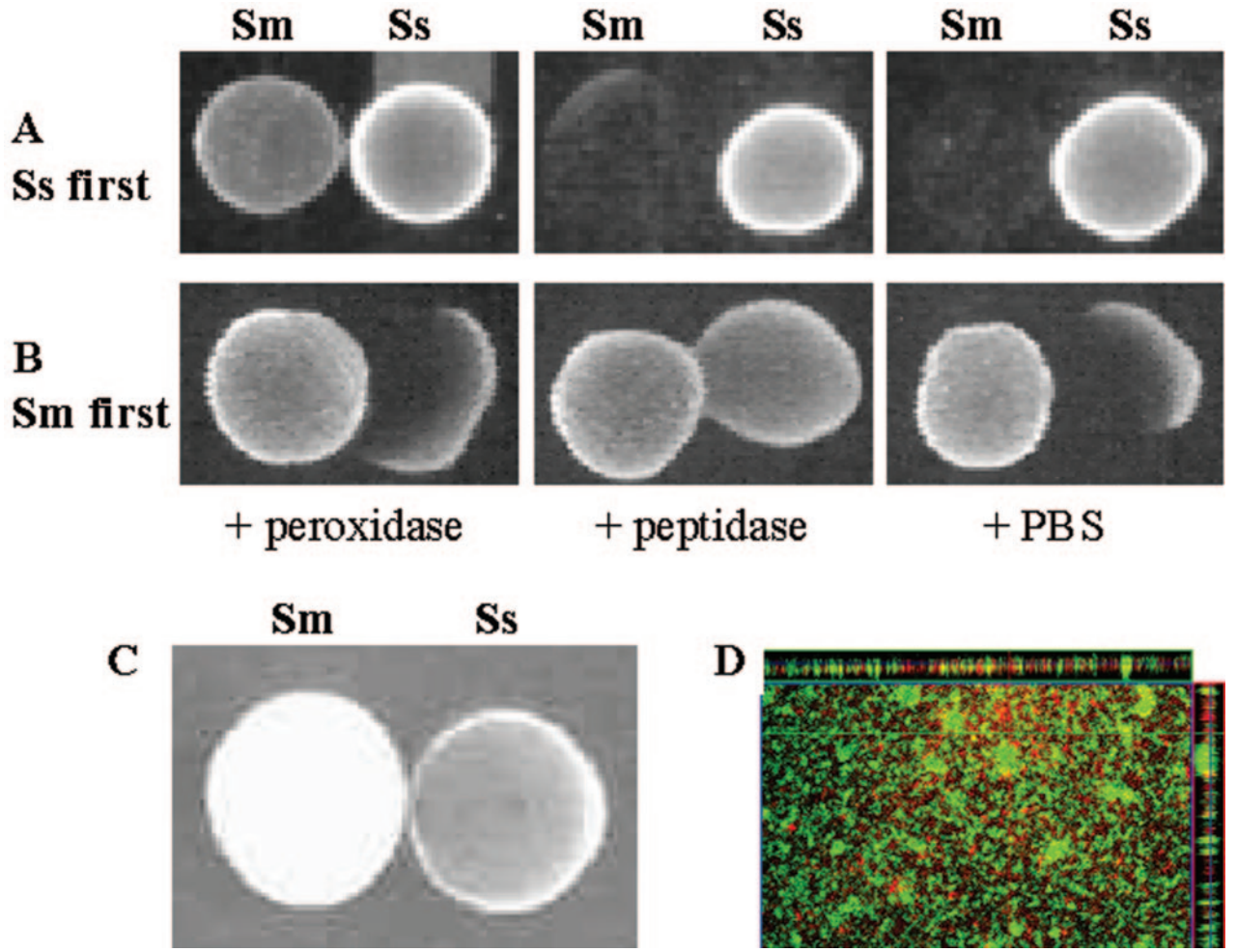

FIG. 3. Identification of inhibitory substances produced by *S. mutans* and *S. sanguinis*. (A) *S. sanguinis* (Ss) was inoculated first. (B) *S. mutans* (Sm) was inoculated first. After 24-h growth on half-strength BHI plates, 40 μg of peroxidase (left), 64 μg of peptidase (middle), or phosphate-buffered saline (right) was added beside the colony before the competing species was inoculated. (C) competition of the mutacin-defective strain UA140I$^-$IV$^-$ with Ss on the plate (C) and in the biofilm (D) when Sm was inoculated first. Green cells, *S. mutans* (green fluorescent protein); red cells, *S. sanguinis* (Cell-tracker orange). The confocal micrograph was taken at ×100 magnification.

ods) to measure H_2O_2 production by *S. sanguinis* and found that under high-cell-density conditions, approximately 120 μM H_2O_2 was produced by *S. sanguinis*, which would be sufficient to affect the growth of *S. mutans*. Although a direct quantification of H_2O_2 production on the plate was not technically feasible, we did observe considerable H_2O_2 production by *S. sanguinis* grown on plates (see Fig. 5C). These data suggested that H_2O_2 produced by *S. sanguinis* could be one of the diffusible inhibitory substances responsible for preventing the growth of *S. mutans*.

To get more direct evidence that these compounds (mutacins and H_2O_2) indeed can carry out the inhibitory effects on *S. sanguinis* and *S. mutans*, respectively, we conducted direct growth inhibition studies. *S. mutans* was challenged with different concentrations of H_2O_2, and growth inhibition was measured (Fig. 4A). The lowest concentration that could inhibit the growth of *S. mutans* was 0.0005% (142 μM), which was in the same range as the H_2O_2 produced by *S. sanguinis* in the cell pellet (see Fig. 6B). Purified mutacin I and mutacin IV were both able to inhibit the growth of *S. sanguinis* in an overlay assay up to an eightfold dilution (Fig. 4B and C). In addition, we conducted overlay assays with the different mutacin mutants. These experiments showed that both mutacins are involved in the *S. sanguinis* growth inhibition and that the double mutant had a dramatically reduced ability to inhibit the growth of *S. sanguinis* (Fig. 4D). These results demonstrate the ability of H_2O_2 and mutacin to inhibit the growth of *S. mutans* and *S. sanguinis*, respectively.

Mutacin gene expression and H_2O_2 production are regulated by growth conditions. To determine the effect of mutacin and H_2O_2 production on the competition outcome between *S. mutans* and *S. sanguinis*, we studied the effect of medium conditions on the production of mutacin and H_2O_2. To quantify mutacin gene expression, we constructed reporter strains in which the promoterless firefly luciferase gene (*luc*) was fused to the mutacin I (*mutA*) and the mutacin IV (*nlmA*) promoters on the chromosome. The reporter strains were inoculated on the three conditioned plates as described in Fig. 2C and D. After 24 h of incubation, the cells were scraped from the plate and measured for luciferase activity and OD_{600}. The spent

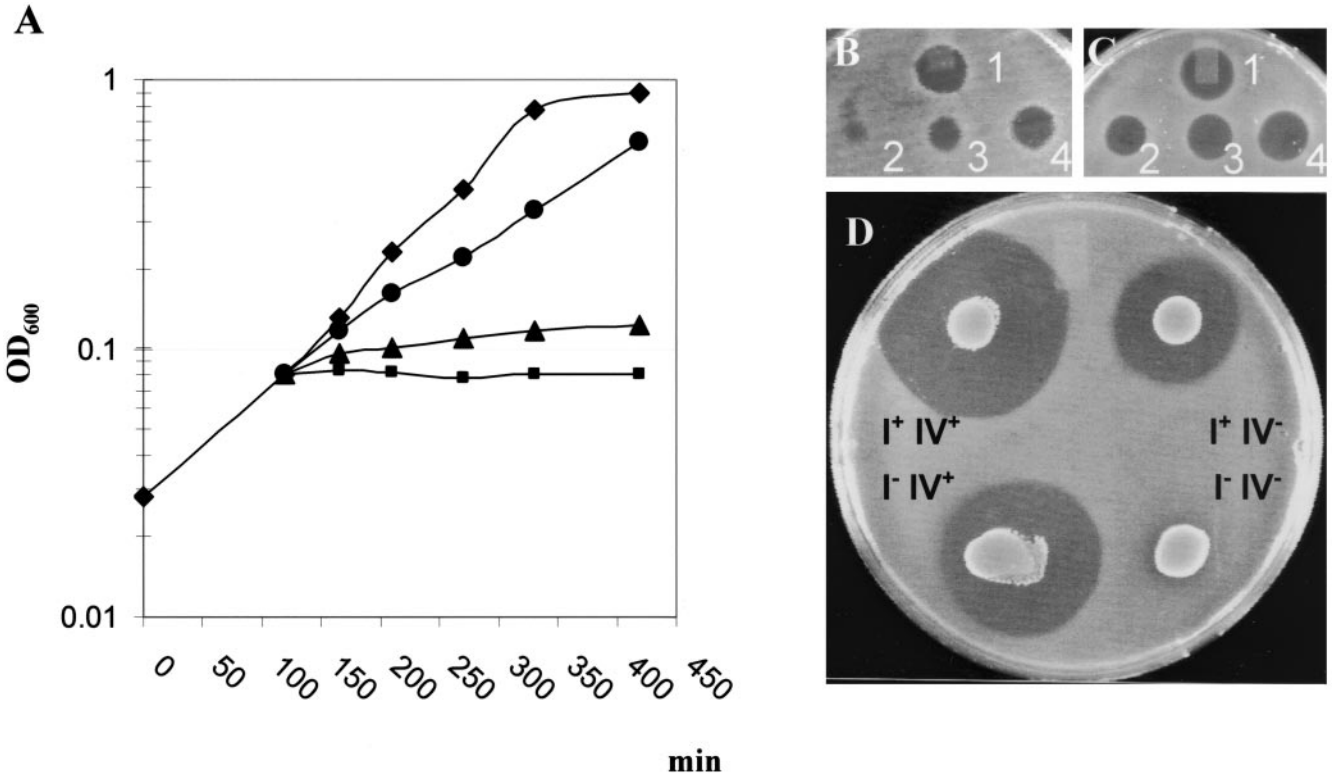

FIG. 4. Effects of H_2O_2, mutacin I, and mutacin IV on the growth of *S. mutans* and *S. sanguinis*. (A) Growth inhibition of *S. mutans* UA140 treated with different concentrations of H_2O_2; ◆, no H_2O_2; ●, 0.0005% (142 μM) H_2O_2; ▲, 0.0025% (710 μM) H_2O_2; ■, 0.005% (1.42 mM) H_2O_2. Experiments were repeated two times with similar results. Shown is a representative result of one experiment. (B and C) Inhibition of *S. sanguinis* with purified mutacin I and partially purified mutacin IV. Different dilutions of purified mutacin I (B) and partially purified mutacin IV (C) were spotted onto a BHI plate and overlaid with *S. sanguinis*. Each spot contained 10 μl of twofold serially diluted extract (i.e., no. 1, undiluted; no. 4, twofold diluted; no. 3, fourfold diluted, etc.). (D) Effects of mutations in mutacin I and mutacin IV genes on the growth of *S. sanguinis*. Overnight cultures of a mutacin I-defective (I^-IV^+), a mutacin IV-defective (I^+IV^-), a double-mutant (I^-IV^-), and a wild-type (I^+IV^+) strain of UA140 were spotted (10 μl) onto BHI plates and overlaid with *S. sanguinis*.

plates were overlaid with an indicator strain to measure mutacin production. Both mutacin I (*mutA*) and mutacin IV (*nlmA*) promoters exhibited the same pattern of expression under these conditions; shown in Fig. 5A and B are the results of the mutacin I promoter expression (*mutAp-luc*) and mutacin I production. Compared to the "nutrient-limiting" plate (bar 1), mutacin I promoter expression was reduced ~10-fold on both "nutrient-rich" (bar 2) and "stress" (bar 3) condition plates (Fig. 5A). Consequently, the inhibition zone on the "nutrient-rich" plate (Fig. 5B, bar 2) was reduced >5-fold compared to that on the "nutrient-limiting" plate (bar 1), and no inhibition zone was observed on the "stress" condition plate (bar 3).

The effect of environmental conditions on H_2O_2 production by *S. sanguinis* was measured on the plate by a modified peroxidase assay (see Materials and Methods). Darker color on and around the colony indicated the presence of larger amounts of H_2O_2. As shown in Fig. 5C, the amounts of H_2O_2 on the "nutrient-rich" (plate 2) and "stress" (plate 3) condition plates were conspicuously less than that on the "nutrient-limiting" (plate 1) plate. Taken together, these results correlated well with the phenotypic observations depicted in Fig. 2.

Mutacin gene expression and H_2O_2 production are both inhibited by juxtaposition between *S. mutans* and *S. sanguinis*. The results presented in Fig. 2A and B demonstrated that despite competitive exclusion between *S. mutans* and *S. sanguinis*, they can coexist under certain circumstances, such as when both species are inoculated at the same time (right). To determine whether close cell-cell proximity between the two species could result in mutual inhibition of inhibitory-substance production by the competing species, we developed a mixed-culture pelleting assay that would create an environment for cell-cell contact but without complications of extensive cell growth. Overnight cultures were diluted and grown to early log phase (OD_{600}, ~0.1), and the two species were mixed in a 1:1 ratio and centrifuged. The mixed cultures were incubated for 2 h as cell pellets before luciferase activity, H_2O_2 production, and OD_{600} were measured. As controls, single-species cultures of *S. mutans* and *S. sanguinis* in planktonic and pelleted conditions were used. Since mutacin I and IV promoters behaved similarly, only the results of mutacin I promoter expression are presented here (Fig. 6). In the single-species culture, mutacin I gene expression increased 10-fold in the cell pellet (bar 2) compared with the planktonic culture (bar 1) (Fig. 6A). Similarly, H_2O_2 production by *S. sanguinis* increased twofold in the cell pellet (bar 2) compared with the planktonic culture (bar 1) (Fig. 6B). These results suggested that high cell density enhanced mutacin gene expression by *S. mutans* and H_2O_2 production by *S. sanguinis*. Surprisingly, in the mixed-

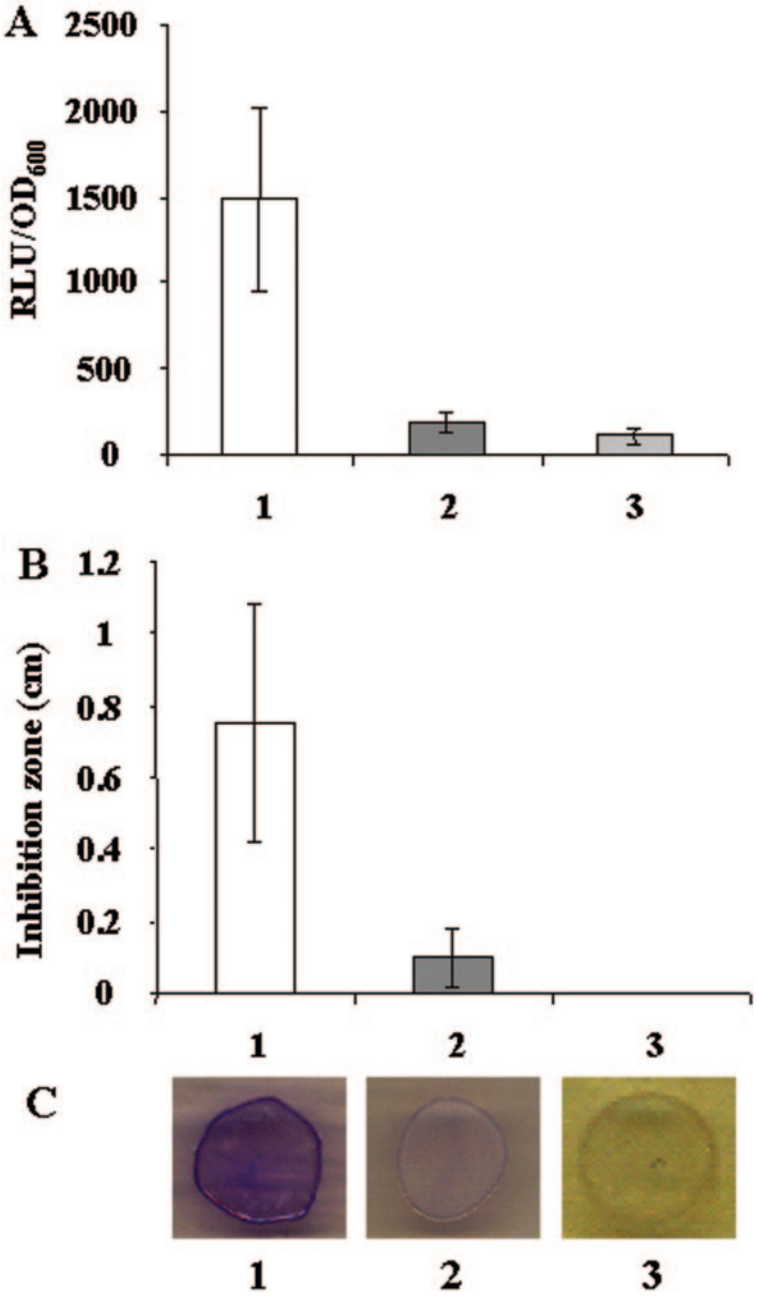

FIG. 5. Effects of growth conditions on mutacin I gene expression (A), mutacin production (B), and H_2O_2 production (C). Mutacin I gene expression (*mutAp-luc*) was measured as relative light units (RLU) per OD_{600} unit; mutacin production was measured by diameters of the inhibition zone against the indicator; H_2O_2 production by *S. sanguinis* was indicated by a purple color (see Materials and Methods). Cells were grown on different conditioned plates: 1, half-strength BHI; 2, BHI plus 1% sucrose, pH 7; 3, BHI, pH 5.5. Presented are representatives of at least two experiments performed on different days (the error bars indicate standard deviations).

species cell pellet, mutacin gene expression by *S. mutans* was reduced fivefold (Fig. 6A, bar 3) and H_2O_2 production by *S. sanguinis* was reduced threefold (Fig. 6B, bar 3) compared with their respective single-species cell pellets.

To further confirm that this inhibition of mutacin gene expression by juxtaposition with *S. sanguinis* happens only between different species, not within the same species, we performed the same pelleting assay with two *S. mutans* strains carrying different fluorescent protein reporters. UA140::Φ(*mutAp-mrfp*) (15) carries a red fluorescent protein fused to the mutacin I promoter, and UA159::Φ(*ldhp-gfp*) carries a green fluorescent protein fused to the *ldh* promoter (16). Pelleting assays were performed with either UA140::Φ(*mutAp-mrfp*) alone (Fig. 6C), UA140::Φ(*mutAp-mrfp*) plus UA159::Φ(*ldhp-gfp*) (Fig. 6D), or UA140::Φ(*mutAp-mrfp*) plus *S. sanguinis* (Fig. 6E). After 2 h of incubation, the cell pellet was analyzed by confocal microscopy. UA140::Φ(*mutAp-mrfp*) cells alone or in a mixture with UA159::Φ(*ldhp-gfp*) exhibited bright-red fluores-

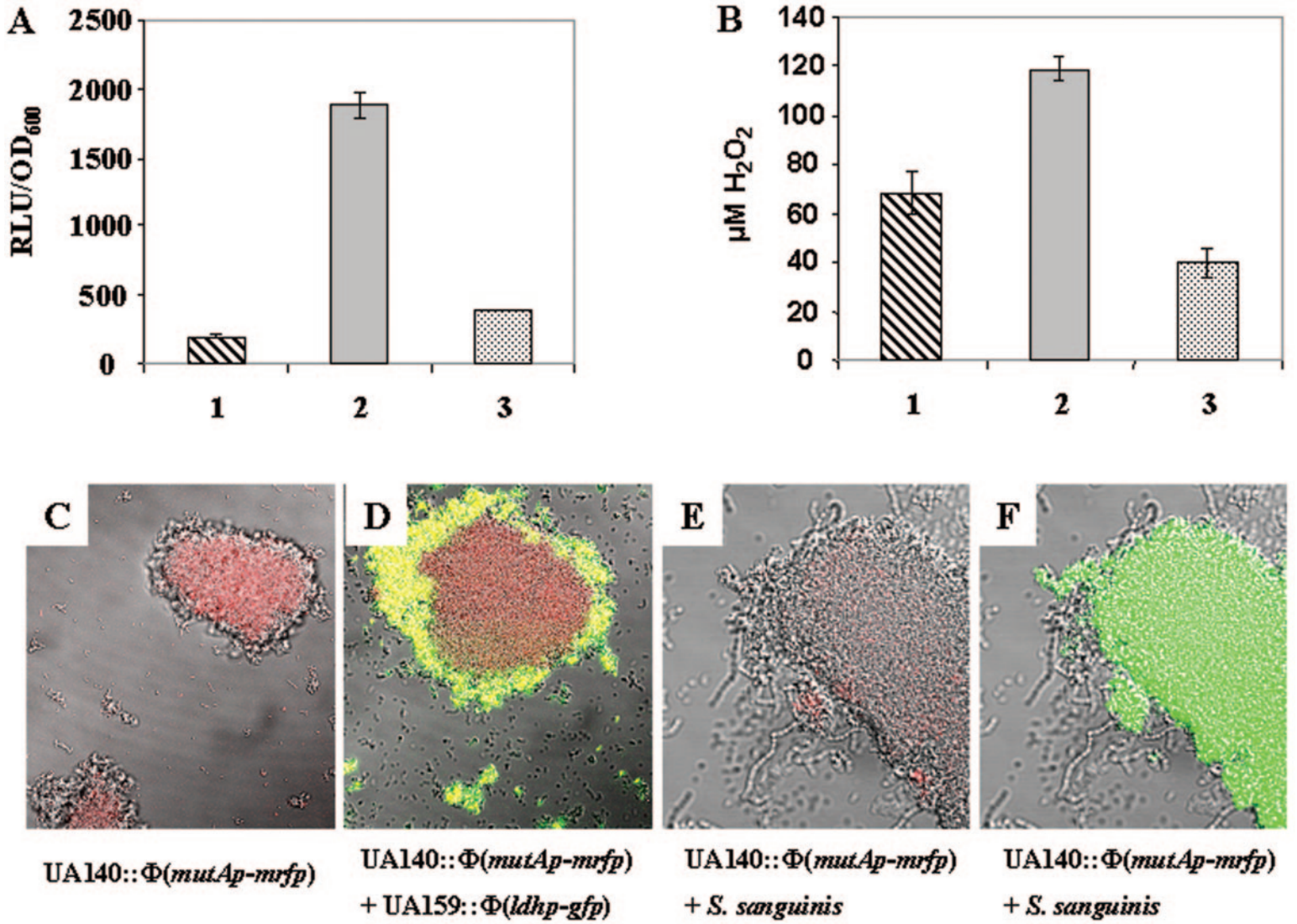

FIG. 6. Effects of juxtaposition between *S. mutans* and *S. sanguinis* on mutacin gene expression and H_2O_2 production. (A) Mutacin I gene expression of strain UA140::Φ(*mutAp-luc*). (B) H_2O_2 production by *S. sanguinis*. Bars 1, single-species planktonic culture; bars 2, single-species pelleted culture; bars 3, mixed-species pelleted culture. The experiment was done three times on different days with similar results. Presented are representative data from one experiment done in duplicate. The error bars indicate standard deviations. (C) Mutacin I gene expression in strain UA140::Φ(*mutAp-mrfp*) single-strain culture. Red fluorescence indicates mutacin I gene expression. (D) Same experiment as in panel C, but mixed with strain UA159::Φ(*ldhp-gfp*). Red, UA140::Φ(*mutAp-mrfp*); green, UA159::Φ(*ldhp-gfp*); yellow, mixture of the two strains. (E) Same experiment as in panel C, but mixed with *S. sanguinis*. (F) Same experiment as in panel E with UA140::Φ(*mutAp-mrfp*) cells labeled with fluorescein isothiocyanate-conjugated anti-*S. mutans* monoclonal antibodies. Green cells, UA140::Φ(*mutAp-mrfp*); gray cells, *S. sanguinis*. The confocal micrographs were taken with fluorescent and differential interference contrast modes.

cence, indicating a high level of mutacin I gene expression; in contrast, the same UA140::Φ(*mutAp-mrfp*) cells displayed almost no fluorescence in the mixed culture with *S. sanguinis* (Fig. 6E). To exclude the possibility that the diminished fluorescence of UA140::Φ(*mutAp-mrfp*) in the mixed-species culture was due to fewer *S. mutans* cells in the cell aggregates, a fluorescein isothiocyanate-conjugated monoclonal antibody specific to *S. mutans* (1) was used to label cells in the mixed-species cell aggregates. As shown in Fig. 6F, similar amounts of UA140::Φ(*mutAp-mrfp*) cells existed in the mixed-species cell aggregates and in the single-species cell aggregates.

To test whether the reduced mutacin gene expression and H_2O_2 production in the mixed-species cell pellet was due to inhibition of cell growth, cells in the single-species and mixed-species cell pellets were plated at the beginning and the end of the experiment. No difference was observed between the single-species and mixed-species cultures, suggesting that the reduced mutacin gene expression and H_2O_2 production in the mixed-species cell pellet was not due to inhibition of cell growth of either species during the 2-h coculturing period (data not shown). To test further whether live cells were required to exert this inhibitory effect, *S. mutans* cells were mixed with UV-killed *S. sanguinis* cells or vice versa, and pelleting assays were performed. Mutacin gene expression or H_2O_2 production was not inhibited when dead cells of the other species were present (data not shown).

Since it could be argued that the pelleting assay created an artificial high-cell-density environment, which may not represent the natural dental biofilm situation, we did another experiment under biofilm conditions. We inoculated UA140::Φ(*mutAp-luc*) as a single-species culture and as a mixed-species culture with *S. sanguinis* in a 1:1 ratio on a BHI plate and incubated the cells for 6 h. Under this biofilm condition, both bacterial species could grow on a surface with an air interface, as could be found in the dental biofilm. The cells were scraped from the plate, and luciferase activity was determined. After normalization with the number of viable cells, we found a 15-fold reduction of the luciferase activity in the mixed-species culture compared to UA140::Φ(*mutAp-luc*) alone (Fig. 7A). As a control, UA140::Φ(*ldhp-luc*) was used to monitor the expression of the housekeeping gene *ldh* (lactate dehydrogenase), which would reflect the metabolic status of the cells (24). As shown in Fig. 7B, the expression of the *ldh* gene remained the same in the mixed-species biofilm as in the single-species biofilm. The increase in the change from 5-fold (in the pellet) to 15-fold (on the plate biofilm) could be explained by the longer incubation time of *S. mutans* in the presence of *S. sanguinis*. The longer incubation time was necessary to yield visible cell growth on the plate. This result further confirmed the observations made in the pelleting assays (Fig. 5), suggesting that in the dental biofilm, the presence of *S. sanguinis* could inhibit *mutA* gene expression of *S. mutans*.

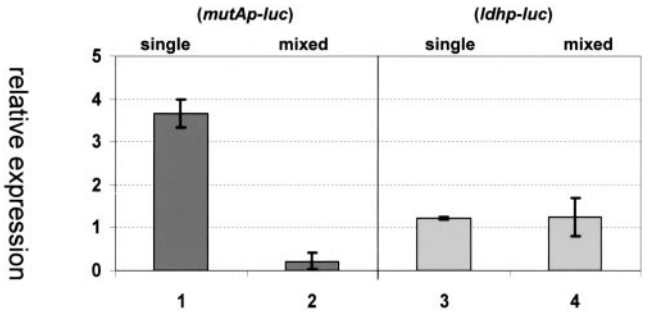

FIG. 7. Relative mutacin I (*mutAp-luc*) and lactate-dehydrogense (*ldhp-luc*) gene expression in single- and mixed-species surface biofilms. Overnight cultures of all strains were adjusted to an OD_{600} of 1. Ten microliters of strain UA140::Φ(*mutAp-luc*) or UA140::Φ(*ldhp-luc*) alone (bars 1 and 3) or mixed in a 1:1 ratio with *S. sanguinis* (bars 2 and 4) were spotted onto a BHI plate. After 6 h of incubation, the cells were scraped from the plate and the luciferase activity was determined. The activity was normalized by the cell counts of *S. mutans* after serial dilution. Experiments were repeated twice with similar results. Shown is a representative result of one experiment done with triplicate samples. (A) Expression of the mutacin I (*mutA*) gene. (B) Expression of the *ldh* gene.

DISCUSSION

A unique feature of the oral biofilm is its high density and diversity of microbial species (9). This high cell density dictates close cell-cell contact within the same species or between different species, which results in inevitable intra- and interspecies interactions. Cooperative interactions among oral bacteria have been well studied. These include coaggregation to facilitate a cell's attachment to the tooth surface (4), nutritional complementation to enable cell growth in saliva (11), and metabolic cooperation between two species (6). These cooperative interactions probably have played very important roles in the development of the dental biofilm; however, antagonistic interactions among different species may be equally important given the conditions in the oral cavity. For example, Xie et al. reported inhibition of *Porphyromonas gingivalis* fimbrial gene expression by *Streptococcus cristatus* mediated by a 59-kDa surface protein (40). In this study, we initiated a systematic investigation of the molecular mechanisms of interspecies competition between *S. mutans* and *S. sanguinis*.

Competition assays on the plate and in the biofilm demonstrated a mutual exclusion between the two species depending on the sequence of inoculation (Fig. 2). This competitive exclusion turned out to be a result of the production of inhibitory substances by the two competing species. Interestingly, when both species were inoculated at the same time, negligible or no competition was observed. Further investigation revealed that when both species were juxtaposed to each other, both mutacin and H_2O_2 production were inhibited (Fig. 6). Since this mutual inhibition was observed only when live cells of the competing species were present in the cell pellet, we speculate that interspecies communications are involved. Further investigations are under way to elucidate the interspecies communication pathways and the molecular signals involved.

Another interesting finding from the competition assays was that on a BHI plate supplemented with sucrose and buffered to pH 7 with phosphate buffer, or on a BHI plate adjusted to pH 5.5 with HCl, competitive exclusion was not observed regardless of which species was inoculated first (Fig. 2C and D). We considered the former condition as "nutrient rich" because sucrose appeared to be the preferred carbohydrate for both species and buffering the medium to pH 7 would prevent inhibition of cell growth by the acids produced during the fermentation of sucrose. Indeed, cells growing on this plate al-

ways achieved higher cell mass than cells growing on a regular BHI plate (data not shown). The BHI plate with pH 5.5 was considered a stress condition because both bacterial species grew more slowly on this plate than on regular BHI plates, although *S. mutans* showed more acid tolerance than *S. sanguinis* (3, 12). How did "nutrient-rich" growth and "stress" conditions suppress competition between the two species? Further studies (Fig. 5) demonstrated that this was achieved at least partially through inhibition of mutacin and H_2O_2 production.

What is the ecological meaning of this environmental modulation of interspecies competition? From a cell's economic point of view, we speculate that it is related to the balance between the cost and benefit of producing mutacins and H_2O_2. Biosynthesis of mutacin I and IV is an expensive process. For mutacin I, at least 11 gene products are required for producing a functional mutacin molecule (32), and for mutacin IV, at least five gene products would be required. Although the exact mechanism of H_2O_2 production by *S. sanguinis* is not known, it also would require energy (13). In this case, mutacin or H_2O_2 production may become a double-edged sword. In a multispecies community, mutacin or H_2O_2 production may give the producer a competitive edge, while it may also slow down the growth of the producers due to the extra energy expenditure. Therefore, it would make perfect ecological sense that when excess nutrient is present, mutacin or H_2O_2 production is shut down to allow more energy to be used for cell growth and species proliferation. Similarly, under stress conditions where cell survival becomes more important than colony expansion, mutacin or H_2O_2 production is also shut off to focus energy expenditure on maintaining the essential cellular functions. Only under conditions where cells have enough energy to compete but not enough food for optimal growth is mutacin or H_2O_2 production activated for competition. These well-regulated strategies may be necessary for the survival and perpetuation of a species in a multispecies community under natural conditions and may be even more so in the oral cavity, where cycles of feast and famine and fluctuations of pH are daily routines. It is also worth noting that mutacin and H_2O_2 production is rather prevalent in clinical isolates of *S. mutans* and *S. sanguinis* (and other members of the mitis group streptococci), respectively (8, 34, 39). So is the competition between *S. mutans* and other oral streptococcal species, as shown in Fig. 1. Therefore, the molecular mechanisms underlying the competition and coexistence between the two species reported in this study may represent a general mechanism underlying interspecies interactions in the dental biofilm.

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